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RESEARCH ARTICLE

Synthesis and biological evaluation of novel N, N′-disubstituted urea and thiourea derivatives as potential anti-melanoma agents

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Abstract

Two series of urea and thiourea derivatives (**1a–11a**, **1b–11b**) have been synthesized; all the 22 compounds were reported for the first time. Their anti-proliferative activities against the melanoma cell line B16-F10 were evaluated. Among the compounds tested, compound **6b** exhibited the most potent activity in melanoma cells growth inhibition (IC_{50} = 0.33 μM). The bioassay tests showed that anti-proliferative activities of these novel compounds were possibly caused by inhibition of ERK1/2 phosphorylation level. Therefore, compound **6b** can be a potential anti-melanoma agent and an inhibitor of ERK1/2 phosphorylation deserving further research.

Keywords: Melanoma, ERK, phosphorylation, urea, thiourea

Introduction

Skin cancer is one of the most common human malignancies and its global incidence is rising at an alarming rate, with basal cell carcinoma, squamous cell carcinoma and melanoma being the most common forms[1]. Due to the rising incidence and lack of effective treatments for advanced disease, melanoma is the most dangerous form, accounting for most skin cancer deaths, so new treatment strategies are urgently needed[2,3]. The growth of melanoma depends on stimulatory effects of various growth factors, which bind to tyrosine kinase receptors to activate various intracellular signalling pathways[1,4]. Recent studies have provided some new therapeutic approaches to this disease. Extracellular signal-regulated kinase (ERK) is one of the most important members of mitogen-activated protein kinase (MAPK) family, which is activated by tyrosine kinase receptors and other upstream signal molecules, such as RAS (RAS-GTP), RAF-1 and MEK1/2[5,6]. The pathway offers several junctures for signal-transduction blockade; due to the converging functions of MEK1/2 and ERK1/2, specific inhibition of these proteins is particularly desirable[7]. The ERK proteins consist of two isoforms (the 44 kDa ERK1 and the 42 kDa ERK2; referred to as ERK1/2)[8,9]. The activities of ERK1/2 are tightly controlled by dual-phosphorylation at special Thr 183/202 and Tyr 185/204 residues[10,11]. The dual-phosphorylated (full-active) forms have determined the structural features required for full-activation[11–13]. Phosphorylated ERK (pERK, active ERK) 1/2 protein transactivates numerous growth-related genes contribute to tumour cell proliferation, tumour cell survival, migration and metastasis[14,15]. This pathway has emerged recently as the central growth-stimulatory pathway in melanoma, with ERK being hyperactivated in up to 90% of human melanomas[1,16,17].

The urea derivatives such as N-nitrosoureas, benzoylureas, thioureas generally represent one of the most useful classes of anticancer agents, with a wide range of activities against various leukemias and solid tumours[18–21]. Our interest in this area is to design and synthesize diverse active urea and thiourea derivatives for anti-tumour agents[22,23]. In continuation of our earlier

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studies, two novel series of N-benzyl-N-(4-hydroxy-3-methoxybenzyl)-N′-phenyl-urea and thiourea (**1a–11a**, **1b–11b**) derivatives were synthesized in this paper, then we found that some of these compounds could inhibit the proliferation of melanoma cell line B16-F10 via block RAS–MEK–ERK signalling pathway.

Experimental procedure Chemistry

All the NMR spectra were recorded on a Bruker DRX 500 or DPX 300 model Spectrometer in $CDCl_3$. Chemical shifts (δ) for ^{1}H NMR spectra were reported in parts per million to residual solvent protons. Melting points were measured on a Boetius micro melting point apparatus. The ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. All chemicals and reagents used in current study were of analytical grade. The TLC was run on the silica gel-coated aluminium sheets (Silica Gel 60 GF254, E. Merck, Germany) and visualized in UV light (254 nm).

General procedure for N-benzyl-N-(4-hydroxy-3-methoxy-benzyl)-N′-phenyl-urea (1a–11a) and thiourea (1b–11b) preparations

The primary substituted amines (12 mmol) was added to a solution of 3-methoxy-4-hydroxybenzaldehyde (10 mmol), in ethanol (30 mL). The mixture was stirred at room temperature, and the reaction was monitored by TLC. The products were filtered and recrystallised in ethanol. Without further purification, to an ethanolic solution of synthesized Schiff bases, $NaBH_4$ (10 mmol) was slowly added in an ice bath with stirring. The mixture was refluxed for 2 h, then the solvent was evaporated and water (20 mL) was added. The product extraction was carried out with CH_2Cl_2 (3 × 20 mL). The organic layer was dried over Na_2SO_4, and after solvent evaporation affording the crude product as yellow oil (**1′**–**11′**). To chloroform solution of amines (**1′**–**11′**), phenylisocyanate or phenylisothiocyanate (1:1) was slowly added with stirring. The mixture was refluxed overnight. The completion of reaction was checked by TLC. The $CHCl_3$ was removed under reduced pressure and the crude product was purified by column chromatography.

Spectral properties of compounds 1a–11a and 1b–11b

1-(4-Hydroxy-3-methoxybenzyl)-1, 3-diphenylurea (1a)

White powder, yield: 75%. Mp: 124°C–126°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 3.83 (s, 3H); 4.80 (s, 2H); 5.57 (s, 1H); 6.61–6.64 (m, 1H); 6.74–7.32 (m, 12H). MS (ESI): 349.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{20}N_2O_3$: C, 72.40; H, 5.79; N, 8.04. Found: C, 72.51; H, 5.97; N, 8.19.

1-(4-Hydroxy-3-methoxybenzyl)-3-phenyl-1-p-tolylurea (2a)

Needle crystals, yield: 79%. Mp: 146°C–148°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 2.37 (s, 3H); 3.84 (s, 3H); 4.81 (s, 2H); 5.54 (s, 1H); 6.61–6.65 (dd, J_1 = 1.83, J_2 = 8.04 Hz, 1H); 6.76–6.78 (d, J = 8.04 Hz, 1H); 6.88–6.89 (d, J = 1.83 Hz, 1H); 6.96–7.31 (m, 9H). MS (ESI): 363.1 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{22}N_2O_3$: C, 72.91; H, 6.12; N, 7.73. Found: C, 72.78; H, 6.05; N, 7.87.

1-(4-Hydroxy-3-methoxybenzyl)-1-(4-methoxyphenyl)-3-phenylurea (3a)

Needle crystals, yield: 60%. Mp: 139°C–142°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 3.80 (s, 3H); 3.85 (s, 3H); 4.81 (s, 2H); 5.56 (s, 1H); 6.62–6.65 (dd, 1H); 6.75–6.78 (d, J = 8.0 Hz, 1H); 6.89–7.23 (m, 8H); 7.27–7.32 (m, 2H). MS (ESI): 379.1 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{22}N_2O_4$: C, 69.83; H, 5.86; N, 7.40. Found: C, 69.79; H, 5.87; N, 7.23.

1-(4-Fluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (4a)

Needle crystals, yield: 74%. Mp: 126°C–128°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 4.80 (s, 2H); 5.57 (s, 1H); 6.60–6.63(dd, J_1 = 2.01, J_2 = 8.04 Hz, 1H); 6.77–6.80 (d, J = 8.04 Hz, 1H); 6.86–6.89 (d, J = 1.83 Hz, 1H); 6.98–7.03 (m, 1H); 7.08–7.24 (m, 6H); 7.27–7.30 (m, 2H). MS (ESI): 367.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}FN_2O_3$: C, 68.84; H, 5.23; N, 7.65. Found: C, 68.90; H, 5.43; N, 7.52.

1-(4-Chlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (5a)

Needle crystals, yield: 67%. Mp: 143°C–145°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 4.81 (s, 2H); 5.58(s, 1H); 6.60–6.62 (dd, 1H); 6.77–6.79 (d, J = 8.0 Hz, 1H); 6.86 (d, J = 1.0 Hz, 1H); 6.98–7.08 (m, 3H); 7.23–7.30 (m, 4H); 7.36–7.38 (d, J = 8.5 Hz, 2H). MS (ESI): 383.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClN_2O_3$: C, 65.88; H, 5.00; N, 7.32. Found: C, 65.91; H, 4.97; N, 7.26.

1-(4-Bromophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (6a)

Crystals, yield: 80%. Mp: 150°C–152°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 4.81 (s, 2H); 5.58 (s, 1H); 6.60–6.64 (dd, J_1 = 1.83, J_2 = 8.03 Hz, 1H); 6.77–6.80 (d, J = 7.8 Hz, 1H); 6.85–6.86 (d, J = 1.83 Hz, 1H); 6.98–7.03 (m, 3H); 7.22–7.31 (m, 4H); 7.51–7.54 (m, 2H). MS (ESI): 427.0 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}BrN_2O_3$: C, 59.03; H, 4.48; N, 6.56. Found: C, 59.16; H, 4.45; N, 6.62.

1-(2-Fluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (7a)

White powder, yield: 43%. Mp: 126°C–129°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 4.76 (s, 2H); 5.65 (s, 1H); 6.57–7.19 (m, 8H); 7.27–7.43 (m, 4H). MS (ESI): 367.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}FN_2O_3$: C, 68.84; H, 5.23; N, 7.65. Found: C, 68.63; H, 5.09; N, 7.75.

1-(2-Chlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (8a)

Needle crystals, yield: 56%. Mp: 117°C–119°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 4.79 (s, 2H); 5.57 (s, 1H); 6.58–7.03 (m, 5H); 7.17–7.34 (m, 7H). MS (ESI): 383.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClN_2O_3$: C, 65.88; H, 5.00; N, 7.32. Found: C, 65.71; H, 5.07; N, 7.51.

1-(2-Bromophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (9a)

White powder, yield: 80%. Mp: 134°C–137°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 4.81 (s, 2H); 5.69 (s, 1H); 6.59–7.51 (m, 12H). MS (ESI): 427.0 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}BrN_2O_3$: C, 59.03; H, 4.48; N, 6.56. Found: C, 60.07; H, 4.59; N, 6.46.

1-(2, 4-Difluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (10a)

White powder, yield: 53%. Mp: 142°C–145°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.83 (s, 3H); 4.76 (d, 2H); 5.62 (s, 1H); 6.60–6.64 (m, 1H); 6.73–7.30 (m, 10H). MS (ESI): 385.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{18}F_2N_2O_3$: C, 65.62; H, 4.72; N, 7.29. Found: C, 65.81; H, 4.75; N, 7.40.

1-(2, 4-Dichlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylurea (11a)

White powder, yield: 46%. Mp: 144°C–145°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.83 (s, 3H); 4.82 (d, 2H); 5.61 (s, 1H); 6.59–6.62 (dd, 1H); 6.77–7.11 (m, 5H); 7.23–7.36 (m, 5H). MS (ESI): 417.1 ([M+H] $^+$). Anal. Calcd for $C_{21}H_{18}Cl_2N_2O_3$: C, 60.44; H, 4.35; N, 6.71. Found: C, 60.52; H, 4.33; N, 6.58.

1-(4-Hydroxy-3-methoxybenzyl)-1, 3-diphenylthiourea (1b)

Needle crystals, yield: 71%. Mp: 136°C–138°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 3.84 (s, 3H); 5.48 (s, 2H); 5.57(s, 1H); 6.64–6.66 (dd, 1H); 6.75–6.77 (d, J = 8.0 Hz, 1H); 6.93 (s, 1H); 7.09–7.10 (m, 2H); 7.16–7.19 (m, 1H); 7.30–7.43 (m, 6H). MS (ESI): 365.4([M+H]$^+$). Anal. Calcd for $C_{21}H_{20}N_2O_2S$: C, 69.20; H, 5.53; N, 7.69. Found: C, 69.13; H, 5.46; N, 7.56.

1-(4-Hydroxy-3-methoxybenzyl)-3-phenyl-1-p-tolylthiourea (2b)

Needle crystals, yield: 65%. Mp: 130°C–132°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm):2.36 (s, 3H); 3.85 (s, 3H); 5.46(s, 2H); 5.56 (s, 1H); 6.63–6.66(dd, J_1 = 1.83, J_2 = 8.07 Hz, 1H); 6.75–6.78 (d, J = 8.07 Hz, 1H); 6.95–6.98 (m, 3H); 7.13–7.34 (m, 7H). MS (ESI): 379.1 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{22}N_2O_2S$: C, 69.81; H, 5.86; N, 7.40. Found: C, 69.85; H, 6.00; N, 7.47.

1-(4-Hydroxy-3-methoxybenzyl)-1-(4-methoxyphenyl)-3-phenylthiourea (3b)

Needle crystals, yield: 67%. Mp: 146°C–148°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.81 (s, 3H); 3.85 (s, 3H); 5.44 (s, 2H); 5.55 (s, 1H); 6.63–6.66 (dd, J_1 = 1.83 Hz, J_1 = 8.04 Hz, 1H); 6.75–6.78 (d, J = 8.04 Hz, 1H); 6.89–7.00 (m, 5H); 7.12–7.20 (m, 1H); 7.27–7.35 (m, 4H). MS (ESI): 395.1 ([M+H]$^+$). Anal. Calcd for $C_{22}H_{22}N_2O_3S$: C, 66.98; H, 5.62; N, 7.10. Found: C, 66.85; H, 5.70; N, 7.26.

1-(4-Fluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (4b)

Needle crystals, yield: 59%. Mp: 136°C–139°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.45 (s, 2H); 5.57 (s, 1H); 6.62–6.65 (dd, 1H); 6.76–6.78 (d, J = 8.04 Hz, 1H); 6.89 (s, 1H); 7.06–7.32 (m, 9H). MS (ESI): 383.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}FN_2O_2S$: C, 65.95; H, 5.01; N, 7.32. Found: C, 66.02; H, 5.03; N, 7.51.

1-(4-Chlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (5b)

Needle crystals, yield: 65%. Mp: 138°C–140°C. ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.45 (s, 2H); 5.58(s, 1H); 6.63–6.65 (dd, J_1 = 1.5 Hz, J_2 = 8.0 Hz, 1H); 6.77–6.78 (d, J = 8.0 Hz, 1H); 6.88 (s, 1H); 7.02–7.04 (d, J = 8.5 Hz, 2H); 7.10–7.11 (d, J = 1.5 Hz, 1H); 7.18–7.21 (m, 1H); 7.31–7.40(m, 5H). MS (ESI): 399.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClN_2O_2S$: C, 63.23; H, 4.80; N, 7.02. Found: C, 63.14; H, 4.86; N, 6.88.

1-(4-Bromophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (6b)

Crystals, yield: 73%. Mp: 150°C–152°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.44 (s, 2H); 5.58 (s, 1H); 6.63–6.66 (dd, 1H); 6.76–6.79 (d, J = 7.86 Hz, 1H); 6.88 (s, 1H); 6.96–6.99 (m, 2H); 7.10–7.11 (d, J = 1.83 Hz, 1H); 7.17–7.21 (m, 1H); 7.30–7.56 (m, 5H). MS (ESI): 443.0 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}BrN_2O_2S$: C, 56.89; H, 4.32; N, 6.32. Found: C, 56.81; H, 4.29; N, 6.24.

1-(2-Fluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (7b)

White powder, yield: 38%. Mp: 133°C–135°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.39 (s, 2H); 5.64 (s, 1H); 6.65–7.05 (m, 4H); 7.16–7.26 (m, 5H); 7.30–7.37 (m, 3H). MS (ESI): 383.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}FN_2O_2S$: C, 65.95; H, 5.01; N, 7.32. Found: C, 65.91; H, 5.06; N, 7.26.

1-(2-Chlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (8b)

Needle crystals, yield: 34%. Mp: 146°C–149°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.43 (s, 2H); 5.58(s, 1H); 6.59–7.38 (m, 12H). MS (ESI): 399.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}ClN_2O_2S$: C, 63.23; H, 4.80; N, 7.02. Found: C, 63.30; H, 4.72; N, 7.16.

1-(2-Bromophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (9b)

White powder, yield: 63%. Mp: 143°C–145°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.31 (s, 2H); 5.49 (s, 1H); 6.57–7.17 (m, 9H); 7.19–7.23 (m, 1H); 7.29–7.32 (m, 2H). MS (ESI): 443.0 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{19}BrN_2O_2S$: C, 56.89; H, 4.32; N, 6.32. Found: C, 56.94; H, 4.22; N, 6.39.

1-(2, 4-Difluorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (10b)

White powder, yield: 45%. Mp: 135°C–137°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.85 (s, 3H); 5.44 (s, 2H); 5.57 (s, 1H); 6.59–6.63 (dd, J_1 = 1.8 Hz, J_2 = 7.86 Hz, 1H); 6.74–6.77 (d, J = 7.86 Hz, 1H); 6.86–7.05 (m, 5H); 7.18–7.23

(m, 1H); 7.29–7.36 (m, 3H). MS (ESI): 401.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{18}F_2N_2O_2S$: C, 62.99; H, 4.53; N, 7.00. Found: C, 62.95; H, 4.48; N, 6.85.

1-(2, 4-Dichlorophenyl)-1-(4-hydroxy-3-methoxybenzyl)-3-phenylthiourea (11b)

White needle crystals yield: 38%. Mp: 125°C–127°C. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 3.83 (s, 3H); 5.39 (d, 2H); 5.60 (s, 1H); 6.60–6.62 (dd, 1H); 6.77–7.21 (m, 7H); 7.32–7.41 (m, 3H). MS (ESI): 433.1 ([M+H]$^+$). Anal. Calcd for $C_{21}H_{18}Cl_2N_2O_2S$: C, 58.20; H, 4.19; N, 6.46. Found: C, 58.07; H, 4.23; N, 6.53.

Cell culture

B16-F10 cells obtained from American Type Culture Collection (ATCC) were cultured in RPMI-1640 medium (GibcoBRL) with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of air/CO_2 (95%: 5%).

Cell proliferation assay

The anti-proliferative activity was determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of 7×10^3 cells/well in 96-well microtiter plates (Costar). After 24 h, exponentially growing cells were exposed to the indicated compounds at final concentrations ranging from 0.1 to 100 μg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (10 μL of 5 mg/mL MTT in PBS). After 4 h, 100 μL of 10% SDS (Sigma) in 0.01 N HCl was added, and the plates were incubated at 37°C for a further 18 h; optical absorbance was measured at 570 nm on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages with respect to untreated cells. The IC_{50} values were determined from replicates of 6 wells from at least two independent experiments.

Cell-based enzyme inhibition measurement by ELISA

Detection of the effect of compounds on p-MEK1/2, t-ERK1/2, pERK1/2, p-Rsk1 and p-Elk-1 activity in B16-F10 cells was performed using ELISA kits (Invitrogen) and strictly according to the manufacturer's instructions. Cells were pre-treated with 30 nM PMA (Sigma) in the absence or presence of these compounds.

Immunofluorescence

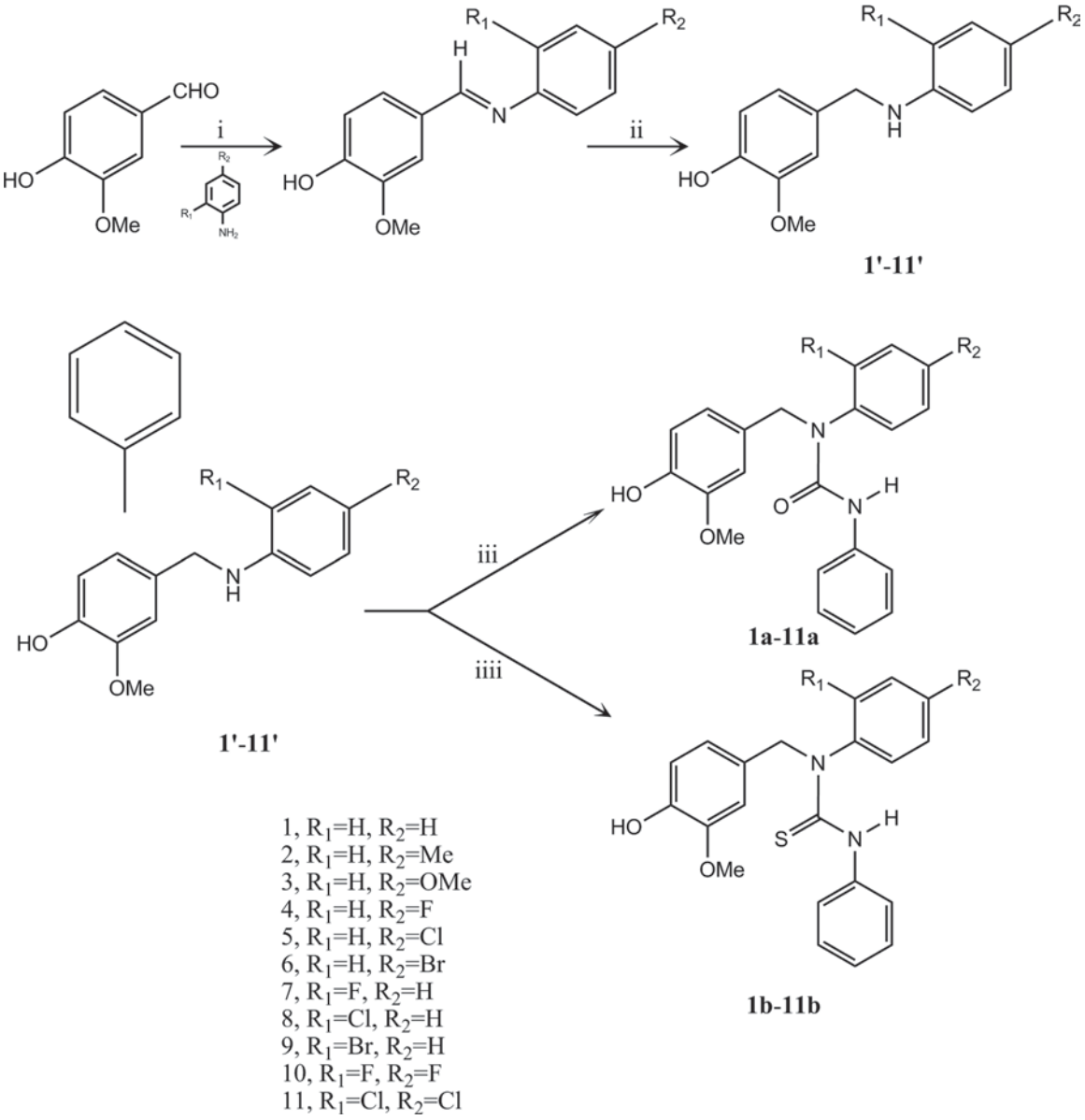
The B16-F10 cells were seeded in 6-well plates at a seeding density of 10^5 cells/mL. When all the cells were adhered, various concentrations of compound **6b** added. For immunofluorescence analysis, B16-F10 cells were treated with PMA (30 nM) and two different concentrations (0 and 0.25 μM) of compound **6b**. After 24 h incubation, cells washed with PBS, fixed with 2% paraformaldehyde for 2h and permeabilized with 0.2% Triton X-100. After blocking with 1% BSA, the cells were incubated for 3h at room temperature with primary anti-pERK1/2 (Boster, 1:100). The cells were incubated with secondary antibodies: FITC-labelled goat anti-rabbit IgG (Boster) at dilution 1:100 for 1h at 37°C in the dark. Slides were analysed under Nikon E800 (Nikon, Japan) microscope.Results and discussion

Chemistry

The synthesis of compounds **1a**–**11a** and **1b**–**11b** followed the general pathway outlined in Scheme 1. The two series of urea and thiourea derivatives were synthesized in three steps. First, we chose 11 different substituted anilines to prepare the corresponding Schiff bases. The secondary amines (**1′-11′**) were obtained from the corresponding Schiff bases after the reduction reactions with sodium borohydride. In the next step, the secondary amines were condensed with phenylisocyanate or phenylisothiocyanate in chloroform as the solvent, affording the target compounds **1a**-**11a** and **1b**-**11b**. The reactions were monitored by thin layer chromatography (TLC) and the products were purified by column chromatography. All the urea and thiourea **1a–11a** and **1b–11b** were synthesized for the first time and all the compounds were fully characterized by ^{1}H NMR, ESI MS and elemental analysis. Furthermore, the crystal data, data collection and refinement parameter for compound **6b** are listed in supplementary information. The structure was solved by direct methods and refined on F2 by full-matrix least-squares methods using SHELX-97[24]. Biological activity study

In vitro cytotoxic activities of these N-benzyl-N-(4-hydroxy-3-methoxybenzyl)-N′-phenylurea and thiourea derivatives were studied in B16-F10 melanoma cell line. A number of synthesized compounds displayed potent cytotoxic activities against B16-F10. The cytotoxic activity of the 22 compounds in B16-F10 was closely associated with their structures, as shown in Table 1 and expressed as the half maximal inhibitory concentration (IC_{50}). All 22 compounds exhibited good activity with IC_{50} values of <10.0 μM, among which 7 compounds demonstrated IC_{50} values of 5.0–10.0 μM, 15 compounds showed IC_{50} less than 5.0 μM, compound **6b** exhibited the most potent inhibitory activity in melanoma cells growth inhibition (IC_{50} = 0.33 μM), which was more potent than positive control cisplatin (IC_{50} = 4.24 μM) and carboplatin (IC_{50} = 7.35 μM).

Structure-activity relationships (SARs) of these urea and thiourea derivatives demonstrated that phenyl thiourea derivatives have less IC_{50} values than those corresponding phenyl urea derivatives. The result also indicated that compounds with substitution at the *para* (**1a**-**6a**, **1b**-**6b**) position showed more potent activities than those with substitution on the *ortho* position (**7a**-**9a**, **7b**-**9b**). A comparison of the *para* position substitution on benzene ring demonstrated that a *para* halogen group (**4a**-**6c**, **4b**-**6b**) may have slightly more improved anti-proliferative activity than a methyl or a methoxy group, and it showed the most potent inhibitory activity when the *para* position was substituted by bromine. A



Scheme 1. Synthesis route of compounds **1a**–**11a** and **1b**–**11b**. Reagents and conditions: (i) ethanol, rt, 4 h; (ii) ethanol, $NaBH_4$, reflux, 2 h; (iii) phenylisocyanate, chloroform, reflux, overnight; (iiii) phenylisothiocyanate, chloroform, reflux, overnight.

Table 1. Cytotoxicity of compounds **1a**–**11a** and **1b**–**11b** against B16-F10 cells.

Compound	R_1	R_2	B16-F10 (IC_{50} ± SD, μM)	Compound	R_1	R_2	B16-F10 (IC_{50} ± SD, μM)
1a	H	H	2.07 ± 0.25	1b	H	H	1.23 ± 0.25
2a	H	Me	4.54 ± 0.97	2b	H	Me	3.39 ± 0.83
3a	H	OMe	5.31 ± 0.61	3b	H	OMe	2.47 ± 0.32
4a	H	F	1.57 ± 0.54	4b	H	F	1.35 ± 0.24
5a	H	Cl	1.49 ± 0.42	5b	H	Cl	1.27 ± 0.36
6a	H	Br	0.99 ± 0.25	6b	H	Br	0.33 ± 0.10
7a	F	H	5.98 ± 1.31	7b	F	H	5.55 ± 0.76
8a	Cl	H	8.96 ± 0.72	8b	Cl	H	6.09 ± 0.36
9a	Br	H	7.95 ± 0.47	9b	Br	H	5.38 ± 0.62
10a	F	F	4.03 ± 1.07	10b	F	F	3.56 ± 0.94
11a	Cl	Cl	3.33 ± 0.63	11b	Cl	Cl	2.57 ± 0.91
Cisplatin			4.24 ± 0.67				
Carboplatin			7.35 ± 0.89				

Table 2. Inhibition (IC_{50}) of pERK1/2 in B16-F10.

Compound	pERK (μM)	Compound	pERK (μM)
1a	6.77	1b	3.35
2a	6.12	2b	5.47
3a	5.76	3b	3.21
4a	4.19	4b	3.12
5a	4.07	5b	3.28
6a	2.14	6b	1.27
7a	9.15	7b	7.22
8a	10.08	8b	6.99
9a	10.17	9b	6.14
10a	6.47	10b	6.92
11a	5.22	11b	4.13

Cells were pre-treated with 30 nM PMA in the absence or presence of these compounds.

significant loss of activity was observed when the halogen substituent was moved to the *ortho* (**7a**-**9a**, **7b**-**9b**) position. This trend was observed in all compounds whether they were urea or thiourea. The compounds (**10a**, **11a**, **10b**, **11b**) substituted by halogen groups both on *para* position and *ortho* position showed more potent activities than compounds with *ortho* halogen substituents, but less than compounds with *para* halogen substituents. So we deduced that substitution at the *para* position of the N-phenyl ring plays an important role in the anti-proliferative activity.

As described above, the ERK signalling pathway plays a central role in several steps of melanoma development, including cancer cell proliferation and the development of resistance to apoptosis. The ERK1/2 is therefore considered a prominent therapeutic target for the development of chemotherapeutic drugs. The synthesized urea and thiourea were evaluated for their ability to inhibit phosphorylation (activation) of ERK in B16-F10 cells. As illustrated in Table 2, all the compounds displayed potent activities to induce the reduction in the amounts of pERK1/2. These results suggested that compounds with substitution at the *para* (**1a**-**6a**, **1b**-**6b**) position showed more potent activities than those with substitution at the *ortho* position, and compound **6b**, which displayed the most potent activity in tumour growth inhibition, also showed the lowest IC_{50} value ($IC_{50} = 1.27$ μM). It is indicated that there is a reasonable correlation between the pERK1/2 inhibitory activities and the cytotoxicities against B16-F10 cells of these compounds. Next, compound **6b** was evaluated for its ability to inhibit pERK1/2 level by using an immunofluorescence analysis[25]. Samples were immunocytochemically labelled with fluorescein isothiocyanate (FITC) for phosphorylated ERK1/2 in green. The results were summarized in Figure 1. Compared to controls, the cells treated with compound **6b** displayed a significant reduction of fluorescence, which verified that this thiourea derivative could inhibit pERK1/2 level in B16-F10 cell line.

As shown in Table 3, we tested the inhibitory activities of selected compounds (**3b**, **4b**, **5b**, **6a**, **6b**) against phosphorylated MEK1/2 and total ERK1/2 (upstream factors

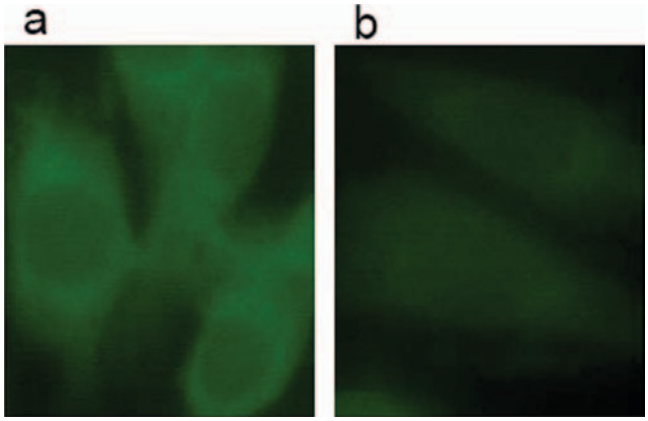


Figure 1. Immunofluorescence staining of pERK1/2 in B16-F10 cells after 24 h incubation. Cells were treated in the absence or presence of **6b**. Green staining indicates the immunofluorescence of human pERK1/2 (FITC-labelled) in B16-F10 cells (a) untreated (b) or treated with 0.25 μM compound **6b**.

Table 3. Inhibition of p-MEK1/2, t-ERK1/2, p-Rsk1 and p-Elk1 in B16-F10 cell line by tested compounds.

	IC_{50} (μM)			
Compounds	p-MEK	t-ERK	p-Rsk1	p-Elk1
3b	> 25	> 25	1.93	2.42
4b	> 25	> 25	2.71	1.95
5b	19.6	> 25	2.76	2.47
6a	> 25	> 25	1.38	1.24
6b	17.8	22.7	0.97	1.53

Cells were pre-treated with 30 nM PMA in the absence or presence of these compounds.

of pERK), phosphorylated Rsk1 and phosphorylated Elk-1 (downstream factors of pERK). These compounds decreased p-Rsk1 and p-Elk-1 amounts (the downstream substrates of ERK1/2) in B16-F10 cells with good effects. However, these compounds did not show obvious inhibitory ability against p-MEK1/2, t-ERK1/2. Combining these enzyme assay results, we deduced that these compounds could specially inhibit the phosphorylation of ERK1/2 and then block the RAS–MEK–ERK signalling pathway in B16-F10 cells.

Conclusion

In summary, two series of novel N-benzyl-N-(4-hydroxy-3-methoxy-benzyl)-N′-phenylurea and thiourea derivatives (**1a**–**11a**, **1b**–**11b**) were synthesized. Their anti-proliferative activities against the melanoma cell line B16-F10 were evaluated. Some compounds displayed good inhibitory activities and the SARs have also been studied. The consequences demonstrated that substitution at the *para* position of the N-phenyl ring played an important role in the anticancer activity. Among the compounds tested, we found compound **6b** had demonstrated significant ERK phosphorylation inhibitory activity ($IC_{50} = 1.27$ μM against pERK1/2) and anti-proliferative activity in melanoma cells growth inhibition ($IC_{50} = 0.33$ μM). In enzyme assays, **6b** did not show

obvious inhibitory ability against upstream factors of pERK1/2. Above all, compound **6b** would be a potential anti-melanoma agent through specially inhibit phosphorylation of ERK1/2.

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Qing-Shan Li and Peng-Cheng Lv have contributed equally to the work.

Declaration of interest

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